

# DEVELOPMENT AND VALIDATION OF CLOPIDOGREL BISULPHATE DETERMINATION IN BULK BY UV SPECTROPHOTOMETRIC METHOD

Lyudmyla Antypenko<sup>1</sup>, Svitlana Gladysheva<sup>2</sup>, Svitlana Vasyuk<sup>3</sup>

<sup>1</sup>Organic and Bioorganic Chemistry Department, Zaporizhzhya State Medical University

<sup>2</sup>Technology of Drugs Department, Zaporizhzhya State Medical University

<sup>3</sup>Analytical Chemistry Department, Zaporizhzhya State Medical University

## ABSTRACT

A simple, rapid, sensitive and low-cost UV-spectrophotometric method has been developed for the quantification of clopidogrel bisulphate in pure substance. It was validated for linearity, range, accuracy, precision, ruggedness and robustness. The valid maximum absorbance was recorded at the 203 nm wavelength in the concentration ranges 1.0-2.6 mg/100mL of clopidogrel bisulphate in methanol solution after 15 min stirring. The limit of detection was found to be 0.59 mg/100mL, while the limit of quantification was 1.78mg/100mL. The calibration curve was linear with  $r^2=0.9929$ . The proposed technique could be successfully used for the determination of clopidogrel bisulphate in bulk in small laboratories without LC-MS spectrometer or HPLC-UV chromatograph, as well as for future pharmacokinetic studies in pharmaceutical dosage forms.

**Keywords:** clopidogrel bisulphate, validation, UV-spectrophotometry

## INTRODUCTION

(+)-(S)-Methyl-2-(2-chlorophenyl)-2-(6,7-dihydrothieno[3,2-c]pyridin-5(4H)-yl)-acetate **A**, namely clopidogrel, is an oral antiplatelet agent used to inhibit blood clots in coronary arteries, peripheral vascular or cerebrovascular diseases and to prevent myocardial infarction (Fig. 1) (1).

Plavix® (clopidogrel), promoted by Sanofi-Aventis and Bristol-Myers Squibb, was the second best-selling drug in the world in 2010 (2). It irreversibly

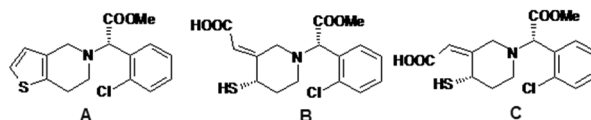


Fig. 1. Structures of clopidogrel (A), and its active metabolites in E- (B) or Z-configuration (C)

inhibits receptor P2Y<sub>12</sub>, an adenosine diphosphate chemoreceptor on platelet cell membranes (1). It is found, that in the first metabolization step the thiophene ring of clopidogrel is subjected to a mono-oxygenation by hepatic cytochrome P450 isozymes (mainly CYP2C19), producing the corresponding 2-oxo-clopidogrel intermediate, a thiolactone, which is further oxidized and undergoes a hydrolytic ring-opening to be finally subjected to a glutathione-dependent reduction giving the active thiol metabolites **B** and **C** (Fig. 1) (3). The augmented antiplatelet effects are expected, when co-prescribed with aspirin, curcumin, cyclosporine, rifampicine, and angiotensin-converting enzyme inhibitor (4). But the low efficacy of clopidogrel is estimated in the presence of

### Address for correspondence:

Lyudmyla M. Antypenko  
Organic and Bioorganic Chemistry Department  
Zaporizhzhya State Medical University  
26 Mayakovsky Ave.  
Zaporizhzhya, 69035  
Ukraine  
e-mail: antypenkol@gmail.com

**Received:** June 26, 2016

**Accepted:** October 7, 2016

esomeprazole, morphine, grapefruit juice, scutellarin, fluoxetine, azole antifungals, calcium channel blockers, sulfonylureas and ritonavir.

According to the Paul W. Elsinghorst review and some others investigations about the quantitative determination of clopidogrel, the predominant analysis techniques were LC-MS, LC-UV, GC-MS and LC-MS/MS, capillary zone electrophoresis coupled with UV detection (3,5). Among drug interaction studies, there were papers about clopidogrel bisulfate and HMG Co-A reductase inhibitors (atorvastatin, pravastatin, simvastatin, rosuvastatin) *via* UV/VIS spectrophotometer at pH 1 or 4 (simulating gastric environments), pH 7.4 (simulating blood pH) and pH 9 (simulating intestinal pH) at body temperature (37°C), or by a reversed-phase liquid chromatographic method (6). Moreover, HPCE and HPLC methods were used for clopidogrel in bulk, for its pharmaceutical dosage forms (7-9). Recently, a sensitive and fast UHPLC-MS/MS detection method was developed for clopidogrel and its active metabolite H4 in human plasma (10). Even X-ray diffraction phase analysis for the mixtures of clopidogrel bisulphate polymorphs was proposed (11). In addition to that, a method based on the oxidation of 2,4-dinitrophenyl hydrazine and coupling of the oxidized product with gemfibrozil and clopidogrel was found, resulting in intensely colored chromogen, which had maximum absorbencies at the 421 nm and 412 nm (12).

Considering the absence of the appropriate apparatuses like mass-spectrometers and HPLC chromatographs, and costs and time-limitations to conduct synthesis in the small analytical laboratories, it was decided to develop a novel low-cost, simple, fast and accurate method of clopidogrel UV detection in the pure substance.

## EXPERIMENTAL PROCESS

### Instrumentation

The substance was weighed using analytical balances Kern ABT 120-5DM, KERN&Sohn GmbH, Germany. Methanol solutions were stirred using ultrasonic cleaning unit Elmasonic E/GB/0605, Elma Hans Schmidbauer GmbH&Co.KG, Germany. UV spectra were recorded on Analytic Jena UV-*vis* spectrophotometer Specord 200 (190-400 nm), Germany.

### Reagents and Solutions

All of the chemicals were of the highest purity available from LAB-SCAN (Ireland) and were used without any further purification. Distilled water was used throughout the experiments. Working substance of clopidogrel bisulphate form II USP (assay by HPLC 100.40% w/w) was purchased from Glochem Industries Limited, India.

### Validation

**Calibration curve.** Standard solution (0.05%) was prepared by dissolving 0.0500 of the clopidogrel in 100.0 mL flask with methanol with thorough stirring for 15 min. Several aliquots of standard solution were taken quantitatively to make the next series of working standard solutions (%): 0.004, 0.0028, 0.0026, 0.0024, 0.002, 0.0018, 0.0016, 0.0014, 0.0012, 0.001, 0.0008, 0.0006, 0.0004, 0.0002 and 0.0001. All solutions were stored at 18–20 °C.

**The calibration curve** of clopidogrel was constructed by UV-visible spectrophotometer absorption data, monitored 5 times for each sample, at the wavelength of maximum absorbance at the 203 nm in 3 mL cuvette with 1cm layer.

**The regression equation** was obtained by the method of least squares for  $n=8$ . Regression equation:  $Y = \text{slope} \cdot x + \text{intercept}$ . Slope, intercept and correlation coefficient were determined from the regression analysis calculations in Microsoft Excel 2007 (13).

Using this linear equation, **correlation coefficient** ( $r^2$ ) and the **detection limits** were determined. **Accuracy:** mean  $\pm$  SD; **Linearity** (lowest – highest concentration while curve is linear); **SE of intercept:**

$$\sqrt{\frac{\sum (Y - Y')^2}{N}}$$

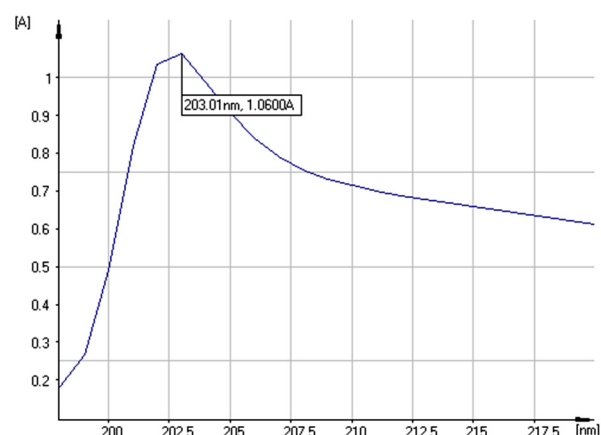
where  $Y$  - standard concentration,  $Y'$  - found concentration; **SD of intercept:** SE of intercept  $\cdot \sqrt{n}$

**The limit of detection (LOD):**  $3.3 \cdot (\text{SD of intercept} / \text{slope})$ ; and the **limit of quantitation (LOQ):**  $10 \cdot (\text{SD of intercept} / \text{slope})$ . The LOD was defined by the concentration with a signal-to-noise ratio of 3. The analyte peak in the LOQ sample should be identifiable, discrete, and reproducible with a precision of  $\pm 20\%$  and accuracy within 80%–120%. The deviation of standards other than LOQ should not be more than  $\pm 15\%$  of the nominal concentration.

**Precision** (repeatability of the method) was evaluated by repeated absorption detection and the results were expressed as the mean standard deviation (SD) and the percent relative standard deviation  $RSD (\%) = SD/Mean$ . For intraday analysis the samples were analyzed six times a day at 09:00 am, 11:00 am, 01:00 pm, 03:00 pm, 05:00 pm, and 07:00 pm, while for interday stability it was analyzed for 6 consecutive days at 09:00 am.

## RESULTS AND DISCUSSION

In the literature data it is reported, that clopidogrel UV-determination followed Beer's law at the 270 nm in the concentration range 5-45 µg/ml in methanol diluted with phosphate buffer (14). It was also estimated at the 219nm in 0.1 N HCl solution with 10-30 µg/ml concentration (15). Cholke PB et al. used triple distilled water pH 1 with 40-70 µg/ml of clopi-



**Fig. 2.** The maximum absorbance at the wavelength of the 203 nm in the UV spectrum of clopidogrel in 0.002% methanol solution

dogrel bisulfate with wavelength of detection 222nm (16).

**Table 1.** Concentration of standard clopidogrel solutions in methanol, their absorbencies, accuracy and recovery data

#	Conc. stand., mg/100 mL	Conc. stand., %	Mean absor- bance of 5 meas.	E, mg/100mL	<sup>b</sup> Conc. found, mg/100mL	<sup>c</sup> Recovery
1	50	0.05	2.0588	24.29	<sup>d</sup> -	-
2	4	0.004	1.7402	2.30	-	-
3	2.8	0.0028	1.3912	2.01	-	-
<sup>e</sup> 4	<b>2.6</b>	<b>0.0026</b>	1.3561	1.92	2.5811	99.2723
5	<b>2.4</b>	<b>0.0024</b>	1.2401	1.94	2.3436	97.64879
6	<b>2</b>	<b>0.002</b>	1.1127	1.80	2.0827	104.1360
7	<b>1.8</b>	<b>0.0018</b>	0.9861	1.83	1.8235	101.3059
8	<b>1.6</b>	<b>0.0016</b>	0.8759	1.83	1.5979	99.8669
9	<b>1.4</b>	<b>0.0014</b>	0.7852	1.78	1.4122	100.8687
10	<b>1.2</b>	<b>0.0012</b>	0.6933	1.73	1.2240	101.9997
11	<b>1</b>	<b>0.001</b>	0.5525	1.81	0.9357	93.5708
12	0.8	0.0008	0.4170	1.92	-	-
13	0.6	0.0006	0.3057	1.96	-	-
14	0.4	0.0004	0.1728	2.31	-	-
15	0.2	0.0002	0.0649	3.08	-	-
16	0.1	0.0001	-0.0378	-2.65	-	-
Mean, n=8					99.8336	
SD					3.1792	
%RSD					2.97886316	
Accuracy (%)					99.8336±3.1792	
Recovery (%)					99.8336±2.9789	

<sup>a</sup> E – percent solution extinction coefficient = Conc. stand., mg/100 mL/Absorbance;

<sup>b</sup> Found concentration: (Absorbance – intercept)/slope;

<sup>c</sup> Recovery: found concentration / labeled concentration\*100;

<sup>d</sup> not tested;

<sup>e</sup> bold values - the concentration range with the best calculated linearity.

In our case, we aimed to conduct the experiment in the conditions that will be closest for the next dialysis investigations (pH about 6,7) with low concentrations of clopidogrel, and easy dilution process and relative cheapness. Different organic solvents like methanol, ethanol, *n*-propanol, isopropanol and *n*-butanol were tested for the investigation. However, the best linear correlation of maximum UV absorbance with concentration was achieved only in a methanol solution.

Analyzing clopidogrel absorbance maximums, two of them were detected in the UV spectrum: the 202-204 and the 270-271nm. Besides, in the investigated concentration ranges (12-14µg/mL) no other suitable absorbance maximums were found. And only at the wavelength of the 203nm the Beer's law was followed. Even if this peak was situated in the spectrum area where it could be overlapped with benzene rings absorbencies of other ingredients, the intent of the given study was development of the fast and low-cost method for pure substance of clopidogrel evaluation (Fig. 2).

Validation of the method was prepared in accordance with the validation parameters of the analytical methods (17).

The fifteen working standard solutions were obtained from the main standard solution (0.05%) and their absorption was measured at the 203nm.

The appropriate percent solution extinction coefficients, accuracy and recovery data in percentage are given in Table 1.

The linearity was evaluated by linear regression analysis, which was calculated by the least-square regression analysis. According to the percent solution extinction and regression coefficients, the calibration curve of clopidogrel with good linearity ( $r^2=0.9929$ ) was found in the concentration range 1.0-2.6 mg/100 mL (solutions 4-11) (Table 2, Fig. 3).

The accuracy of the method was proven by calculating the recovery at eight different concentrations of the calibration range marked with bold numbers in the Table 1 ( $99.83\pm 3.18\%$ ). The mean percentage of recoveries was found to be  $99.98\pm 2.98\%$ .

The presented curve could be used for simple and fast evaluation of unknown clopidogrel sample quantity after appropriate dilution in methanol.

Table 2. Linearity data, accuracy and precision of clopidogrel in methanol

Parameters	Clopidogrel data
Slope	0.4884
Intercept	0.0955
Linearity (mg/100 mL)	1.0-2.6
Regression equation	$y = 0.4884x - 0.0955$
$r^2$	0.9929
SE of intercept	0.0307
SD of intercept	0.0869
LOD (µg/100 mL)	0.5875
LOQ (µg/100 mL)	1.7802

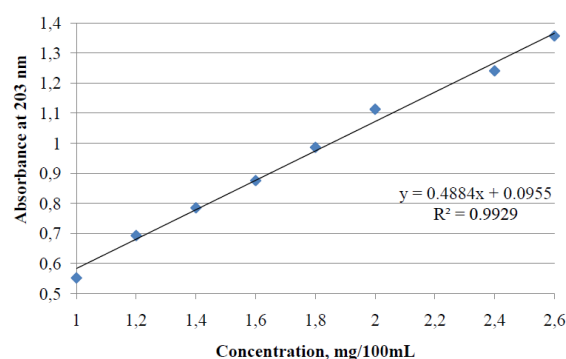


Fig. 3. Calibration curve of clopidogrel

Slope, intercept and correlation coefficient were determined from the regression analysis calculations (Table 2).

The limit of detection (LOD) was found to be  $0.59\mu\text{g}/100\text{mL}$ , while the limit of quantification (LOQ) was  $1.78\mu\text{g}/100\text{mL}$ .

The ruggedness of the method was determined by performing the same assay by different scientists and performing the assay on different days to check the reproducibility. The results were found to be highly reproducible during the day – RSD was 0.83%, but during the week it deteriorated to 4.12% (Table 3).

To determine the robustness of the method, experimental conditions like room temperature, absence of stirring or addition of ultrasound, as well as different methanol series were checked. It was found that usage of the cuvette without glass cap, due to methanol evaporation, affected the results negatively. Furthermore, it should be noted that without stirring clopidogrel bisulphate was distributed in the methanol very unevenly. Ultrasound stirring caused clopi-

**Table 3.** Intraday and interday precision data of 0.002% clopidogrel methanol solution

#	Absorbance	
	Intra-day precision	Inter-day precision
1	1.0644	1.0644
2	1.0664	1.0214
3	1.0622	1.0118
4	1.0695	0.9951
5	1.0589	0.9542
6	1.0423	0.9427
Mean	1.0606	0.9983
SD	0.0097	0.0450
%RSD	0.8324	4.1164

dogrel destruction, because final results were of lower values. So, ordinary physical stirring and temperature of 20°C were the most important factors to obtain accurate data.

Hence, to detect the amount of clopidogrel bisulphate in pure substance the next method is proposed.

0.0500 of clopidogrel bisulphate was quantitatively placed in the 100.0mL flask, dissolved with methanol to obtain 0.05% solution and stirred for 15min. Then 2.00mL of the obtained solution was quantitatively transferred in the 50.0mL flask and methanol was added to obtain 0.002% one and stirred for 15min. 7.00mL of the solution was quantitatively transferred in the 10.00mL flask, methanol was added to obtain the final 0.0014% solution, then stirred for 15 min. The amount of clopidogrel bisulphate was determined by employing UV absorption at the wavelength of 203nm in comparison to methanol in 3mL cuvette with 1 cm layer.

The sample concentration was calculated in accordance with the next equation:

$$C, \% \text{ of final solution} = \frac{A_i - 0.0955}{0.4884 \cdot 1000},$$

where  $A_i$  – absorbance of the investigated sample in methanol at the 203 nm in 3 mL cuvette with 1 cm layer;

Or, in comparison to the measured absorbance of standard solution:

$$C, \% \text{ of final solution} = \frac{A_i \cdot 0.0014}{0.7793}$$

where  $A_i$  – absorbance of the investigated sample in methanol at the 203 nm in 3 mL cuvette with 1 cm layer;

0.0014 - concentration (%) of the standard solution with absorbance of 0.7793 at the 203 nm (calculated according to the obtained regression equation). or concentration in the initial sample:

$$C, \% \text{ (w/V) in bulk} = \frac{A_i \cdot C_0 \cdot 100.0 \cdot 50.0 \cdot 10.00}{A_0 \cdot 2.00 \cdot 7.00 \cdot l \cdot a}$$

where  $A_i$  – absorbance of the final experimental sample solution;

$C_0$  – concentration of the clopidogrel sulphate standard solution is 0.0014, %;

100.0, 50.00, 10.00 – flasks dilutions volume, mL;

$A_0$  – maximum absorbance of 0.0014% standard solution at the 203 nm is 0.7793,

2.00, 7.00 – sample volume taken by pipette, mL;

$l$  – cuvette layer, 1 cm;

$a$  – sample weight, g.

## CONCLUSIONS

It was found, that in a methanol solution clopidogrel bisulphate could be simply, at a low-cost, fast and accurately qualitatively and quantitatively determined by UV spectroscopy in the pure substance by the maximum absorbance at the 203nm. Validation of the proposed method showed, that the calibration curve had good linearity ( $r^2=0.9929$ ) in the concentration range 1.0-2.6mg/100mL. The LOD was found to be 0.59mg/100mL, and LOQ was 1.78mg/100mL. Such criteria like accuracy, precision, robustness and ruggedness also showed high validity and reproducibility of the method, noticing the high importance of ordinary stirring and 20°C temperature. The proposed technique could be successfully used for the determination of clopidogrel bisulphate in the conditions of the smallest laboratories without LC-MS spectrometer or HPLC-UV chromatograph in the pure substance, as well as for the development of nov-



el methods of pharmacokinetic studies in the pharmaceutical dosage forms.

## REFERENCES

1. Ferri N, Corsini A, Bellosta S. Pharmacology of the new P2Y<sub>12</sub> receptor inhibitors: insights on pharmacokinetic and pharmacodynamic properties. *Drugs*. 2013, 73, 1681-1709.
2. Clopidogrel [Internet]. 2016. [updated 2016 May 27; cited 2016 Jun 02]. Wikimedia Foundation, Inc. Available from: <https://en.wikipedia.org/wiki/Clopidogrel>.
3. Elsinghorst PW. Quantitative determination of clopidogrel and its metabolites in biological samples: a mini-review. *J Chromatogr B Anal Technol Biomed Life Sci*. 2013, 917-918, 48-52.
4. Wang ZY, Chen M, Zhu LL, Yu LS, Zeng S, Xiang MX, et al. Pharmacokinetic drug interactions with clopidogrel: updated review and risks management in combination therapy. *Ther Clin Risk Manag*. 2015, 11, 449-467.
5. Vocilkova L, Opatrilova R, Sramek V. Determination of Clopidogrel by Chromatography, *Curr Pharm Anal*. 2009, 5(4), 424-431.
6. Sultana N, Arayne MS, Nawaz M, Ali KA. Zero-crossing derivative spectrophotometry and liquid chromatographic method for the quantitative determination of clopidogrel in presence of HMG Co-A reductase inhibitors. *J Chil Chem Soc*. 2013, 1, 1584-1589.
7. Kumar VP, Sunandamma Y. Simultaneous determination of clopidogrel and pioglitazone by high performance liquid chromatography in bulk drug and dosage forms. *Int J Pharma Life. Sci*. 2013, 2(1), 1-9.
8. Javed MK, Iqbal Z, Khan A, Khan A, Shah Y, Ahmad L, et al. Development and validation of HPLC-UV method for the determination of clopidogrel in pharmaceutical dosage form and human plasma. *Chromatogr Relat Technol*. 2011, 34, 2118-2129.
9. Karaźniewicz-Łada M, Danielak D, Głowska F. HPCE and HPLC methods for determination of clopidogrel and its carboxylic acid metabolite in biological samples: a comparative analysis. *J Liq Chromatogr R T*, 2014, 37(5), 620-633.
10. Hua W, Lesslie M, Hoffman BT, Binns C, Mulvana D. Development of a sensitive and fast UHPLC-MS/MS method for determination of clopidogrel, clopidogrel acid, and clopidogrel active metabolite H4 in human plasma. *Bioanalysis*. 2015, 7(12), 1471-1482.
11. Uvarov V, Popov I. Development and metrological characterization of quantitative X-ray diffraction phase analysis for the mixtures of clopidogrel bisulphate polymorphs. *J Pharma Biomed Analysis*, 2008, 46(4), 676-82.
12. Srujana S, Chandan RS, Gurupadayya BM, Raagaleena V, Manoja KML. Validated Spectroscopic Determination of Clopidogrel and Gemfibrozil Using 2, 4-Dinitro phenyl hydrazine Reagent. *J Pharm Res*. 2012, 5(4), 1906-1909.
13. Etheridge D. Microsoft Office Excel 2007 Data Analysis: Your Visual Blueprint for Creating and Analyzing Data, Charts, and PivotTables. 3<sup>rd</sup> ed. Indianapolis, Indiana: Wiley Publishing; 2007.
14. Patil LD, Gudi SV, Jadav DD, Kadam, YA Dalvi SD, Ingale PL. Development and validation of UV-spectrophotometric methods for simultaneous estimation of amlodipine besylate and clopidogrel bisulfate in bulk and tablet dosage form. *Der Pharma Chemica*. 2012, 5(4), 282-287.
15. Gurav S, Venkatamahesh R. Development and Validation of Derivative UV-Spectrophotometric Methods for Quantitative Estimation of Clopidogrel in Bulk and Pharmaceutical Dosage Form. *Int. J. Chem. Tech. Res*. 2012, 4(2), 497-501.
16. Cholke PB, Ahmed R, Chemate SZ, Jadhav KR. Development and Validation of Spectrophotometric Method for Clopidogrel bisulfate in pure and in film coated tablet dosage form. *Arch. Appl. Sci. Res.*, 2012, 4 (1), 59-64.
17. Guidance for industry, bio-analytical method validation [Internet]. 2001. [updated 2016 Jun 10; cited 2016 May 25]. <http://www.fda.gov/downloads/Drugs/GuidanceComplianceRegulatoryInformation/Guidances/ucm070107>.